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# COMPARATIVE EFFICACY OF INOCULATION METHODS TO STUDY PATHOGENICITY AND VIRULENCE OF DIFFERENT ISOLATES OF *RALSTONIA SOLANACEARUM* INCITING BACTERIAL WILT IN BRINJAL PLANTS

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## ABSTRACT

**Bacterial wilt disease incited by *Ralstonia solanacearum* (*Smith*) is a devastating plant disease that is responsible for heavy losses to the large number of economic host plants it infects. Bacterial wilt of brinjal is severe problem in Chhattisgarh state. This study was carried out for better understanding of biological habit and virulence of various isolates of *Ralstonia solanaceearum* collected from eight brinjal growing districts of Chhattisgarh and attempt was made to find out efficient inoculation method in brinjal plants for proving pathogenicity and virulence. Considerable variability in virulence of different isolates of *R. solanacearum* were found and assessed by comparing per cent wilt incidence produced by respective isolates under the artificial inoculation condition in pot culture study. Isolate collected from Balod district (I-8) appeared as most virulent among other isolates as it produced severe wilting in brinjal plants. Isolate I-1 was not able to produce much wilting and observed as least virulent as compared to other isolates of *R. solanacearum*. Initially wilt symptom produced by most of isolates were started appearing on 7$^{th}$ day of inoculation in brinjal plants and subsequently remain severe till 30$^{th}$ DAI. On comparing the efficacy of different inoculation methods, significantly highest bacterial wilt incidence (94%) with typical wilting symptom and uniform disease development was found in case of soil drenching method followed by root dipping + soil drenching (83%), root dipping (72%), stem injection (60%) and remain least in leaf clipping method (52%) of inoculation at 30 DAI.**

**(Keywords: Brinjal, bacterial wilt, *R. solanacearum*, pathogenicity, virulence, inoculation methods)**

## INTRODUCTION

In tropical and subtropical region of the world, bacterial wilt in brinjal (*Solanum melongena* L.), caused by *Ralstonia solanacearum (Smith) (syn Pseudomonas solanacearum EF Smith)*, is one of the most economically important soil-borne disease. *Ralstonia solanacearum* is gram negative plant pathogenic bacterium that causes lethal wilt disease in more than 450 plant species belonging to monocotyledonous and dicotyledonous plant families (Alladi *et al*., 2014; Elphinstone 2005; Genin 2012; Hayward 1991; and Anusha *et al.,* 2014). It was first described by Smith (1896) in potato, tomato, and eggplant. Bacterial wilt causes great losses because of its severe symptoms, wide geographic distribution, and unusually broad host range, which includes more than 200 plant species belonging to 53 different families. This pathogen enters plant roots through wounds and multiplies rapidly in the vascular system. Rapidly multiplying bacterial cells clog the xylem elements and further block the xylematic flow, leading to fast drooping of foliage followed by wilting and eventually plant death.

The yield loss in India due to this disease has been estimated up to 10-90 per cent (Singh *et al*., 2012). Due to diverse biological habit of *Ralstonia solanacearum*, eradication of pathogen is not possible but can be managed. Various management strategies have been employed such as use of chemicals, host plant resistance, organic soil amendments, biological control, alterations in the date of planting, crop sanitation etc. Resistance to this disease is rarely observed. Therefore, present study was carried out for better understanding of biological habit and virulence of various isolates of *Ralstonia Solanaceearum* collected from different brinjal growing districts of Chhattisgarh and attempt was made to find out efficient inoculation method in brinjal plants for proving pathogenicity and virulence.

## MATERIALS AND METHODS

Experiments were carried out during 2019-20 in pot culture under shed net house at centre of excellence for

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**Collection of wilted samples**

Bacterial wilted samples of brinjal plants were collected from different vegetables growing districts of Chhattisgarh.The collected plant samples were packed in polythene bags and kept at $4^0$ C for isolation of causal organism.Bacterial ooze test were performed for the confirmation of bacterial symptoms and it's presence.

**Isolation, purification and preparation of bacterial inoculum**

A total of 8 *R. solanacearum* isolates were obtained from the wilted brinjal plant samples collected from different districts of Chhattisgarh State. *R. solanacearum* inoculum was prepared from the bacterial colonies obtained from freshly wilted brinjal plants. The bacterium was isolated by streaking a loopful of flowing ooze containing the bacteria on to sterile TTC (Triphenyl Tetrazolium Chloride) agar media (Kelman, 1954) and plates were incubated at 28-30$^0$C for 48-72 hours. After incubation the virulent *R. solanacearum* colonies (irregular shape, white to cream colour, slimy pink colour in the centre) were selected, purified on CPG agar medium and preserved at-80$^0$C in glycerol (30%) for further studies. The bacterial inoculum was further mass multiplied in CPG broth and concentration was adjusted to an optical density of 0.3 at 600 nm wavelength (about $1\times10^8$ cfum $l^{-1}$).

**Nursery raising**

The bacterial wilt susceptible seedlings of brinjal (Pusa purple long) were raised on coco peat in portrays and 20-25 days old (2-3 leaves stage) seedlings were used for transplanting in pots under shed net conditions. Before inoculation, the plants were starved for 24 hours by avoiding watering.

**Inoculation of *R. solanacearum* suspension**

Five different methods of inoculation, *viz*., root dipping, soil drenching, root dipping + soil drenching, stem injection and leaf clipping methods were used for inoculating the brinjal seedlings with each 8 different isolates of *R. solanacearum* suspension taken for study.

**1. Root dipping method**

In this method, roots of uprooted seedlings were washed with water and placed the seedlings into 250 ml beaker filled with 100 ml of bacterial suspension with a certain concentration ($1x10^8$ cfum $l^{-1}$ inoculum). Prior to transplanting lateral tertiary roots of seedlings were injured or cut with a sharp scissor and dip in the above bacterial suspension for 30 minutes. The inoculated seedlings were transplanted into pots filled with disease free soil. The bacterial wilt symptoms were observed in a period of 2-4 weeks.

**2. Soil drenching method**

In this method, one week after transplanting, pots filled with disease free soil were inoculated with 50 ml of bacterial suspension ($1x10^8$ cfum $l^{-1}$ inoculums concentration) by soil drenching. One-third root system of each cultivar was slightly injured by inserting a sharp sterilized knife about 2 cm away from the stem prior to drenching to facilitate penetration of the bacterium (Aslam *et al*., 2017).

**3. Root dipping + soil drenching method**

In this method, both above mentioned methods were used for inoculation, initially brinjal seedlings were inoculated by dipping the roots in bacterial suspension followed by soil drenching.

**4. Stem injection method**

This method was carried out by using thin and sharp microinjection, through which bacterial suspension was injected in the stem base and leaf axil region.

**5. Leaf clipping method**

Leaf clipping method was carried out by dipping the sterile scissors in the bacterial suspension ($1x10^8$ cfum $l^{-1}$ inoculums concentration) and clipping 3-4 leaves of 45 days old brinjal plants per seedling by giving horizontal cut.

The whole experiment was carried out under controlled condition in completely randomized block design (CRD) with 5 replications each with 20 plants. Eight isolates of *R. solanacearum* suspensions (Table. A) were inoculated by above mentioned methods. Pathogenecity of all isolates were proved and their virulence based on different inoculation methods were assessed by calculating wilting percentage. Most virulent isolate in different methods of inoculation was recorded. Periodical observations were made on bacterial wilt symptom expression and wilting percentage was recorded in all the five methods of inoculations after 7, 15 and 30 days of inoculation.

$$\% \text{ Wilt incidence} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}} \times 100$$

# RESULTS AND DISCUSSION

Brinjal plants grown were evaluated against bacterial wilt disease under shed net house in pot culture and data on per cent wilt incidence were recorded, to carried out pathogenicity and confirmation test of different isolates, a infected stem section was cut from the plant with vascular discoloration using a sharp knife or blade. Milky white strands containing bacteria and extracellular polysaccharide streamed out from the cut ends of the xylem.

Data presented in Table1 revealed that, in root dip method of inoculation isolate I-8 has been found as most virulent and showed highly significant wilting incidence i.e. 62%, 65% and 72% at 7, 15 and 30 DAI, which was followed by isolate I-7 (50%, 53%, 60%) and isolate I-5 (36%, 41%, 48%) at 7, 15 and 30 DAI., whereas isolate I-1 was least virulent among all at 7, 15 and 30 DAI. Plants were severely wilted after 30 DAI by isolate I-8 and isolate I-7. Isolate I-1 was not shown any wilt symptoms at 7 DAI and produced only 2% wilting at 30 DAI.

Inoculation of brinjal plants with soil drenching method (Table 2) also revealed that significantly maximum

wilting incidence was observed in plants inoculated by isolate I-8 (85%, 90%, 94%), followed by isolate I-7(64%, 70%, 76%) and isolate I-5 (49%, 55%, 62%) after 7, 15 and 30 days of inoculation. Isolate I-1 showed minimum incidence only, 1-3% witling was recorded after 7, 15 and 30 days of inoculation.

In root dip + soil drench method (Table 3) also, plants inoculated with isolate I-8 showed highest and significant wilting incidence after 7, 15 and 30 days of inoculation i.e. 73%, 76% and 83% respectively, Followed by isolate I-7 and isolate I-5 i.e. 56%, 59%, 67% and 41%, 44%, 53% of wilting after 7, 15 and 30 days of inoculation. Isolate I-1 showed minimum incidence only 2% witling was recorded at 7 and 15 days after inoculation, whereas it increased only 3% of wilting at 30 DAI.

Data presented in Table 4 showed that, in stem injection method isolate I-8 was found most virulent with wilting incidence of 52%, 55% and 60% at 7, 15 and 30 DAI, followed by Isolate I-7 (40%, 42%, 49%), isolate I-6 ( 28%, 31%, 36%), isolate I-5 (16%, 19%, 24%) and isolate I-4 (6%, 9%, 14%). Wilting incidence of isolate I-3 didn't differ significantly with isolate I-2 and incidence of both isolates remained at par. Isolate I-1 was least virulent among all other isolates at 7, 15 and 30 DAI. It did not produce any symptom after 7 days of inoculation and wilting incidence recorded was only 1% at 30 DAI.

In leaf clipping method (Table 5), isolate I-8 showed highly significant wilting incidence i.e. 8%, 45% and 52% at 7, 15 and 30 DAI, followed by Isolate I-7 with 7%, 35% and 72% wilting incidence at 7, 15 and 30 days of inoculation respectively. Wilting incidence recorded in plants inoculated with other isolates were I-6 (6%, 15% and 24%), I-5 (5%, 25%, 37%), I-4 (3%, 5%, and 10%), however, wilting incidence produced by the plants on inoculation with isolate I-3 remain at par with isolate I-4 and I-2 at 7, 15 and 30 days of inoculation. No symptom of wilting has produced by plants inoculated with isolate I-1 even after 30 days of inoculation therefore isolate I-1 remained least virulent among all other isolates.

After the pathogenicity of all the eight isolates have been proved by following different inoculation methods, isolate collected from Balod district (I-8)appeared as most virulent. Therefore, wilt incidence of isolate I-8 were used for comparing efficacy of different inoculation methods. Data given in Table 6 showed that the soil drenching method of inoculation was found most efficient among all other methods. The inoculation through soil drenching method recorded significantly higher bacterial wilt incidence of 85%, 90% and 94% after 7, 15 and 30 days of inoculation respectively, followed by wilt incidence recorded from plants inoculated through root dipping+ soil drenching (73%, 76%, 83%) and root dipping method (62%, 65%, 72%) at 7, 15 and 30 DAI. Wilt incidence recorded via stem injection method of inoculation was comparatively less i.e. 52%, 55% and 60% at 7, 15 and 30 DAI. However, least wilting incidence was recorded from plants inoculated by leaf clipping method at 7 DAI, 15 DAI and 30 DAI i.e. 41%, 45% and 52 %, respectively and appears to be less effective than other methods of inoculation. Soil drenching method of inoculation showed higher degree of wilting from 7- 30 DAI as compare to other methods and started producing wilting symptom in brinjal plants during initial weeks of inoculation, whereas wilting occurred on inoculation through leaf clipping method was least even after 30 DAI.

In accordance to result of our experiment, Sharma and Singh (2019), also performed experiment for assessment of effective inoculation method in tomato plant and recorded highest bacterial wilt incidence (91.33%) with typical wilting symptom in case of soil drenching method followed by leaf clipping method (52%) and stem inoculation method (39.33%), over control.The results are also in accordance with the findings of Artal *et al*. (2012) as they reported that the inoculation through soil drenching method recorded significantly highest bacterial wilt incidence. Zakir Hussain *et al*. (2005) also found that soil drenching method of inoculation was ideal for wilt incidence in brinjal and observed the wilt symptom in brinjal after 20 days of inoculation.Experimenting with tomato for the development of bacterial wilt resistant varieties, Hanson *et al*. (1996) also concluded that the soil drenching method of inoculation was ideal in getting maximum per cent of wilt incidence in tomato.On the contrary, Napoleon *et al*. (2017) concluded that stem inoculation method was more effective than soil drenching method while, assessing pathogenic ability of *Ralstonia pseudo solanacearum* (*Ralstonias olanacearum* phylotype I) from ornamental Rosa spp. and revealed that stem inoculation after wounding resulted in higher disease severity and disease incidence than soil drenching method.

The results of pathogenicity test revealed that all the collected isolates of *R. solanacearum* were able to cause wilt symptoms in brinjal plants. After proving the pathogenicity of all the isolates, it was concluded that all isolates were found virulent throughout the experiment. Considerable variability in virulence of different isolates of *R. solanacearum* were found and assessed by comparing per cent wilt incidence produced by respective isolates under the artificial inoculation condition in pot culture study. Isolate I-8 was most virulent among other isolates as it produced severe wilting in brinjal plants. Isolate I-1 was not able to produce much wilting and observed as least virulent as compared to other isolates of *R. solanacearum*.

Results also revealed that all methods of inoculation produced bacterial wilt symptoms with variable and non uniform pattern, except soil drenching in which all inoculated plants showed most typical, reliable and uniform bactetrial wilt of vascular tissue disease development. The disease mainly affected brinjal plants showing wilting with drooped leaves and necrosis symptoms. Typical symptoms were first observed in soil drenching method. The first symptom appeared on youngest leaves which are the first to be affected on 7$^{th}$ day of inoculation and subsequently severe till 30$^{th}$ DAI. As the disease advanced most of plants

collapsed on 30th day of inoculation.Highest bacterial wilt incidence (94%) with typical wilting symptom and uniform disease development was found in case of soil drenching method followed by root dipping + soil drenching (83%), root dipping (72%), stem injection (60%) and least in leaf clipping method (52%) of inoculation.

Therefore, it is concluded that the best method of inoculation for screening brinjal plants for bacterial wilt disease is the soil drenching method, which is less cumber some, reliable and effective as it exactly stimulates the natural infection as the bacterium is soil borne and enters the plant through roots under field condition (Kelman and Sequeira, 1965; Schmit, 1978; Vasse *et al*., 1995).

**Table A. Different isolates of *R. solanacearum* collected from various districts of Chhatisgarh-**

| Isolates | Districts | Host |
|---|---|---|
| **I-1** | Bilaspur | Brinjal |
| **I-2** | Raipur | Brinjal |
| **I-3** | Durg | Brinjal |
| **I-4** | Kawardha | Brinjal |
| **I-5** | Mahasamund | Brinjal |
| **I-6** | Bemetara | Brinjal |
| **I-7** | Balodabazar | Brinjal |
| **I-8** | Balod | Brinjal |

**Table 1.Wilt incidence in brinjal plants by root dipping method**

| | Wilt incidence (%) | | |
|---|---|---|---|
| **ISOLATES** | **7 DAI** | **15 DAI** | **30 DAI** |
| **I-1** | 0(0) | 1(2.58) | 2(5.16) |
| **I-2** | 3(7.74) | 4(10.33) | 6(14.01) |
| **I-3** | 14(21.79) | 18(25.04) | 23(28.61) |
| **I-4** | 7(15.12) | 9(17.32) | 13(21.03) |
| **I-5** | 36(36.73) | 41(39.76) | 48(43.80) |
| **I-6** | 23(28.35) | 28(31.6) | 36(36.670 |
| **I-7** | 50(44.98) | 53(46.72) | 60(50.76) |
| **I-8** | 62(51.97) | 65(53.81) | 72(58.15) |
| SE(m) | 1.978 | 2.098 | 1.838 |
| C D at 5% | 5.720 | 6.070 | 5.318 |

Figures in parentheses are arcsin values

**Table 2.Wilt incidence in brinjal plants by soil drenching method**

| | Wilt incidence (%) | | |
|---|---|---|---|
| **ISOLATES** | **7 DAI** | **15 DAI** | 30 DAI |
| **I-1** | 1(2.58) | 2(5.16) | 3(7.74) |
| **I-2** | 8(16.22) | 9(17.32) | 12(19.82) |
| **I-3** | 28(31.88) | 29(32.48) | 32(34.39) |
| **I-4** | 18(24.97) | 20(26.41) | 22(27.92) |
| **I-5** | 49(44.4) | 55(47.89) | 62(51.94) |
| **I-6** | 38(38.03) | 42(40.35) | 47(43.25) |
| **I-7** | 64(53.14) | 70(56.84) | 76(60.85) |
| **I-8** | 85(67.3) | 90(72) | 94(77.43) |
| SE(m) | 1.484 | 2.006 | 2.163 |
| C D at 5% | 4.295 | 5.804 | 6.26 |

Figures in parentheses are arcsin values

**Table 3.Wilt incidence in brinjal plants by Root dipping + soil drenching method**

| | Wilt incidence (%) | | |
|---|---|---|---|
| **ISOLATES** | **7 DAI** | **15 DAI** | **30 DAI** |
| **I-1** | 2(5.16) | 2(5.16) | 3(6.26) |
| **I-2** | 5(12.91) | 6(14.01) | 8(15.99) |
| **I-3** | 11(19.06) | 13(21.03) | 16(23.41) |
| **I-4** | 19(25.61) | 22(27.67) | 28(31.83) |
| **I-5** | 41(39.76) | 44(41.52) | 53(46.70) |
| **I-6** | 29(32.45) | 33(34.98) | 39(38.61) |
| **I-7** | 56(48.44) | 59(50.21) | 67(55.17) |
| **I-8** | 73(58.69) | 76(60.66) | 83(65.67) |
| S E (m) | 1.82 | 1.86 | 2.15 |
| C D at 5% | 5.27 | 5.37 | 6.23 |

Figures in parentheses are arcsin values

**Table 4.Wilt incidence in brinjal plants by stem injection method**

| | Wilt incidence(%) | | |
|---|---|---|---|
| **ISOLTES** | **7 DAI** | **15 DAI** | **30 DAI** |
| **I-1** | 0(0) | 1(2.58) | 0(0) |
| **I-2** | 1(2.58) | 2(5.16) | 2(5.16) |
| **I-3** | 2(5.16) | 3(7.74) | 4(8.85) |
| **I-4** | 6(14.01) | 9(17.32) | 14(21.9) |
| **I-5** | 16(23.53) | 19(25.73) | 24(29.25) |
| **I-6** | 28(31.88) | 31(33.77) | 36(36.82) |
| **I-7** | 40(39.2) | 42(40.37) | 49(44.4) |
| **I-8** | 52(46.12) | 55(47.86) | 60(50.76) |
| S E (m) | 1.667 | 2.04 | 1.926 |
| C D at 5% | 4.823 | 5.903 | 5.573 |

Figures in parentheses are arcsin values

**Table 5.Wilt incidence in brinjal plants leaf clipping method**

| | Wilt incidence (%) | | |
|---|---|---|---|
| **ISOLATES** | **7 DAI** | **15 DAI** | **30 DAI** |
| **I-1** | 0(0) | 0(0) | 0(0) |
| **I-2** | 1(2.58) | 1(2.58) | 1(2.58) |
| **I-3** | 2(5.16) | 3(7.74) | 8(10.33) |
| **I-4** | 3(10.33) | 5(11.43) | 10(18.19) |
| **I-5** | 5(26.41) | 25(29.89) | 37(33.77) |
| **I-6** | 6(18.19) | 15(22.66) | 24(26.48) |
| **I-7** | 7(33.72) | 35(36.23) | 72(40.37) |
| **I-8** | 8(39.74) | 45(42.10) | 52(46.13) |
| S E (m) | 2.106 | 1.987 | 1.625 |
| C D at 5% | 6.093 | 5.751 | 4.702 |

Figures in parentheses are arcsin values

**Table 6. Comparison between different inoculation methods**

| Inoculation methods | Wilt incidence (%) | | |
|---|---|---|---|
| | 7 DAI | 15 DAI | 30 DAI |
| **Root dipping** | 62(51.97) | 65(53.81) | 72(58.15) |
| **Soil drenching** | 85(67.30) | 90(72.00) | 94(77.43) |
| **Root dipping + Soil drenching** | 73(58.69) | 76(60.66) | 83(65.67) |
| **Stem injection** | 52(46.12) | 55(47.86) | 60(50.76) |
| **Leaf clipping** | 41(39.74) | 45(42.10) | 52(46.13) |
| S E (m) | 2.366 | 2.28 | 2.145 |
| C D at 5% | 7.03 | 6.774 | 6.372 |

Figures in parentheses are arcsin values

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